6 Temperature and the circadian clock

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6.1 Introduction

Plants are remarkably adaptable to ecological stresses such as pathogen attack, shading from a nearby plant, drought, or temperature. Each of these stresses elicits a cascade of signaling events within the plant, resulting in physiological responses naturally selected to maximize growth and fitness. These signaling events are coordinated with the daily light–dark and warm–cold cycles present on Earth. Perhaps one of the most startling examples of plant preparedness is the anticipation of tomorrow. For example, a sunflower plant will reposition its leaves at the end of the night to face the east in anticipation of dawn to exploit light absorption at the onset of dawn (Shell and Lang 1976) (http://plantsinmotion.bio.indiana.edu/plantmotion/movements/tropism/solartrack/solartrack.html). This remarkable ability to anticipate the arrival of sunlight and subsequent warming is dependent on an internal biological clock. This biological clock is essential for synchronizing diurnal environmental changes such as temperature, a major signal affecting clock function (McClung and Davis 2010). This chapter will focus on addressing the current understanding of the relationship between temperature and the circadian clock in plants.

The first experiments describing circadian rhythms were reported in 1729 by the French astronomer de Mairan who observed that plant leaves exhibit daily rhythmic movements that persist in constant darkness (de Mairan 1729). This innate diurnal periodicity was measured a century later by de Candolle and others and found to be approximately, but not exactly, 24 h in length (de Candolle 1832). These astute observations revealed an internal mechanism within the plant that was entrained to the 24 h day and night cycle present on Earth. The term circadian, coined in 1959 (Halberg et al. 1959) from the Latin words 'circa' (about) and 'dies' (day), is used to describe the 24 h period that signifies one complete cycle of these biological rhythms.

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An endogenous circadian clock is present in all three domains of life, eubacteria, archaea, and eukaryotes. This includes cyanobacteria, halobacteria, fungi, plants, invertebrates, and vertebrates such as mice and humans (Bell-Pedersen et al. 2005; Foster and Roenneberg 2008; Whitehead et al. 2009; Edgar et al. 2012). As humans, the most noticeable workings of our internal clock are our sleep patterns. The desire to sleep when it is dark and be awake when it is light is one manifestation of the orchestrated entrainment of our metabolism, physiology, and behavior to the Earth's rotation (Sahar and Sassone-Corsi 2012). Although the molecular components of the circadian clock across biological taxa are quite different (Bell-Pedersen et al. 2005; Zhang and Kay 2010), the defining characteristics of circadian rhythms are conserved. Circadian rhythms display three fundamental properties: self-sustaining periodicity, entrainment, and temperature compensation (Harmer et al. 2001). Self-sustaining periodicity refers to the persistence of a circadian rhythm under constant conditions, first demonstrated by de Mairan for leaf movement in constant darkness (DD). A true circadian rhythm can be entrained to environmental cues, termed *zeitgeber* ('time giver' in German). The strongest *zeitgeber* is the light/dark (LD) cycle, although temperature cycles also are effective *zeitgebers* (Harmer et al. 2001). The final property of circadian rhythms, and one focus of this chapter, is temperature compensation, whereby the period remains relatively constant at varying physiologically relevant temperatures.

The widespread occurrence of an endogenous circadian oscillator across diverse taxa suggests that there is a fitness advantage in entraining internal processes to the external environment. In plants, the circadian clock is involved in a vast array of processes, from stomatal conductance and photosynthesis to flowering time, stem elongation, leaf movement, resistance to microbial pathogen invasion, and even herbivory resistance (Yakir et al. 2007; Resco et al. 2009; Goodspeed et al. 2012). The importance of the clock in plants is evident in the breadth of circadian regulation of gene expression. Transcriptomic analyses have shown that genes known to be involved in most, if not all, facets of plant growth are circadian regulated (Doherty and Kay 2010). These studies predict between 6% and 90% of the transcriptome in the model plant *Arabidopsis thaliana* is circadian regulated, depending on the condition examined (Harmer 2009; Doherty and Kay 2010). Evidently, the environmental conditions and the methods used to assess the level of transcriptomic regulation by the clock provide different estimates; however, there is no question as to the involvement of the clock in transcript accumulation. In *Arabidopsis*, proper clock regulation of growth confers a fitness advantage compared to long- and short-period mutants whose endogenous periods do not resonate with the environmental cycle (Dodd et al. 2005b; Resco et al. 2009; Yerushalmi and Green 2009).

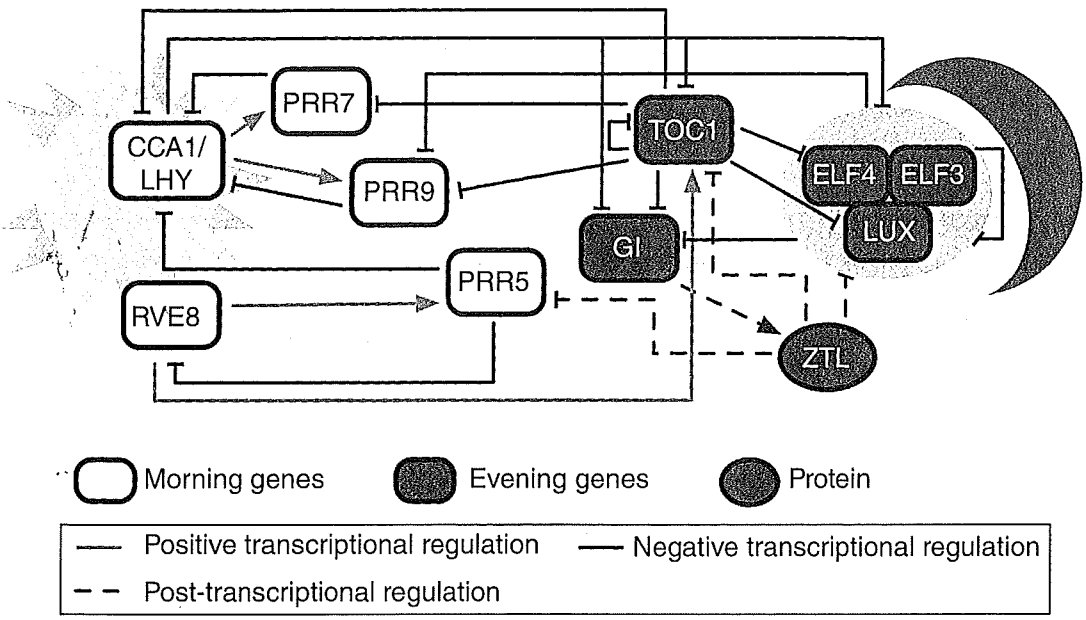

Figure 6.1 Model of the circadian clock in *Arabidopsis*. Due to the complexity of the circadian clock in *Arabidopsis*, a simplified version is shown to emphasize factors currently known to be involved in temperature responses. Transcriptional regulation is emphasized in this model and depicted by *solid* lines. *Green* and *black* lines represent positive and negative regulation, respectively. The *yellow* blocks are genes expressed during the day; *blue* blocks represent evening-expressed genes. *Dashed* lines indicate posttranscriptional regulation with ZTL, the only protein depicted in the model. The EC is represented by the *light blue* circle surrounding *ELF3*, *ELF4*, and *LUX*. Lines that meet the blue circle indicate regulation of the entire complex, whereas lines leading to specific blocks indicate regulation of that gene alone. For color detail, please see color plate section.

This competitive advantage has also been demonstrated in cyanobacteria where mutant strains with altered circadian period outcompete wild-type strains when grown in a culture where the LD cycle length matches the period of the mutant (Ouyang et al. 1998; Woelfle et al. 2004).

A common feature of circadian oscillators in all eukaryotes is the presence of negative transcriptional feedback loops. The circadian clock in plants is comprised of a series of interlocked regulatory feedback loops that can be simplistically divided into a central loop coupled to a morning and an evening loop, defined by the timing of expression of their components (Figure 6.1). The 'morning loop' consists of two MYB transcription factors, CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), whose expression peaks at dawn (Schaffer et al. 1998; Wang and Tobin 1998). The arrhythmic phenotypes caused by overexpression of CCA1 or LHY were important indicators that these proteins are vital components of the clock (Barak et al. 2000). Both CCA1 and LHY activate the expression of two additional morning loop components, PSEUDO-RESPONSE REGULATOR (PRR) proteins PRR7 and PRR9 (Farré et al. 2005; Nakamichi et al. 2005, 2010). PRR9,

PRR7, and another member of the family, PRR5, complete the loop by repressing the expression of *LHY* and *CCA1* (Harmer 2009; Nakamichi et al. 2010). The expression of *PRR5* is positively regulated by REVEILLE8 (RVE8), an myb-like transcription factor closely related to CCA1/LHY (Rawat et al. 2011).

The PRR family is made up of five members, PRR9, PRR7, PRR5, PRR3, and PRR1/TIMING OF *CAB* EXPRESSION1 (TOC1). TOC1 is a component of the 'evening loop' along with the evening complex (EC) that includes EARLY FLOWERING 3 (ELF3), ELF4, and LUX ARRHYTHMO (LUX) (Helfer et al. 2011; Nusinow et al. 2011). The EC is a recent addition to the evening loop even though the necessity of each member for maintaining plant circadian rhythms has been known for some time (Hicks et al. 1996a; Doyle et al. 2002; Hazen et al. 2005; Kolmos et al. 2009). Although LUX is the only member of the complex shown to bind DNA directly, both ELF3 and ELF4 are important for proper expression of the EC transcriptional targets (Kolmos et al. 2009; Helfer et al. 2011; Nusinow et al. 2011; Herrero et al. 2012).

Apart from transcriptional circuitry, there is substantial posttranslational regulation in the clock network. Both TOC1 and PRR5 protein levels are regulated through an SCF-dependent degradation mediated by the F-box protein ZEITLUPE (ZTL) (Harmer 2009; Somers and Fujiwara 2009). ZTL protein stabilization is dependent on another protein, GIGANTEA (GI) (Somers et al. 2000; Han et al. 2004; Kim et al. 2007). GI was originally identified by the extremely late-flowering phenotype of several *gi* mutant alleles compared to wild type (Rédei 1962; Koornneef et al. 1991), although subsequent studies revealed that GI has an important role in the circadian oscillator as well (Fowler et al. 1999; Park et al. 1999). The *GI* transcript shows rhythmic expression under (LL) light conditions, and this pattern is disrupted in genotypes with defective circadian clocks, such as *CCA1* and *LHY* overexpression lines (Fowler et al. 1999). The stabilizing effect of GI on ZTL confers a circadian rhythm in ZTL protein abundance, even though ZTL transcript does not oscillate. In addition, the release of accumulated ZTL from GI at dusk causes a rapid increase in the rate of TOC1 and PRR5 degradation (Kim et al. 2007).

There are several layers of negative regulation between the morning and evening loops. The regulation of *CCA1/LHY* by TOC1 is supported by abundant genetic data, including altered expression of *CCA1*, *LHY*, and *GI* following TOC1 overexpression (Pruneda-Paz and Kay 2010; Somers 2012). Characterization of the TOC1 protein has revealed DNA-binding capability and transcriptional-repression activity (Gendron et al. 2012; Huang et al. 2012; Pokhilko et al. 2012; Somers 2012). TOC1, as shown by chromatin immunoprecipitation, binds to the promoters and represses the

expression of the morning elements *CCA1*, *LHY*, *PRR9*, and *PRR7*, as well as members of the evening loop, *GI*, *TOC1*, *ELF4*, and *LUX* (Huang et al. 2012), revealing extensive feedback regulation within and between the core loops.

The EC also regulates the morning loop by repressing *PRR9* as well as negatively regulating its own expression (Kolmos et al. 2009; Dixon et al. 2011; Helfer et al. 2011; Chow et al. 2012). Similarly, the morning loop interacts transcriptionally with the evening loop through the negative regulation of CCA1/LHY on *TOC1* (Alabadí et al. 2001; Ding et al. 2007), *LUX* (Hazen et al. 2005), *ELF4* (Kikis et al. 2005; Li et al. 2011), and *GI* (Locke et al. 2005). *TOC1* expression is also regulated through an H3 acetylation-dependent process that is antagonized by CCA1. H3 acetylation at the Evening Element (EE) motif in the *TOC1* promoter leads to a relaxed chromatin structure, allowing activation of transcription (Perales and Más 2007; Más 2008). Figure 6.1 depicts a slightly modified version of a recent model of the clock network in *Arabidopsis* (Pokhilko et al. 2012); although this model incorporates a large amount of data, it is simplified for clarity.

The model shown in Figure 6.1 highlights the complexity of the transcriptional and posttranscriptional regulation of the circadian clock genes. This complex network is important for integrating signals from environmental and physiological cues. As mentioned at the beginning of this chapter, the clock controls important physiological, metabolic, and behavioral processes. The clock functions as a central integrator by relaying environmental signals to the necessary output pathways for a coordinated growth response. A good example of this clock-dependent coordination is the regulation of hypocotyl growth. Light, hormones, and temperature are some of the pathways that regulate hypocotyl elongation (Vandenbussche et al. 2005; Nozue and Maloof 2006). Many clock mutants show defects in hypocotyl elongation at least in part due to the inability to properly gate the growth period (Zagotta et al. 1996; Schaffer et al. 1998; Más et al. 2003; Nozue and Maloof 2006). Under LD conditions hypocotyl elongation occurs toward the end of the night cycle, a growth period that is defined by the EC (Nozue et al. 2007; Niwa et al. 2009; Nusinow et al. 2011). Therefore, the clock acts as a point of integration of these various signals to properly gate the hypocotyl growth response.

Another important environmental signal in the clock mechanism is temperature signaling, as marked by the inherent properties of temperature compensation and entrainment of the clock. A tremendous amount of work has led to the comprehensive model of the clock we have today; however, we still know very little about how the plant senses changes in temperature in terms of either daily fluctuations or seasonal effects (McClung and Davis 2010).

6.2 Temperature compensation

The fourth assessment report released in 2007 by the Intergovernmental Panel on Climate Change (IPCC) projects that low-latitude regions, where most global cereal production occurs, are at risk for yield reduction with a temperature increase of as little as +1°C and almost certainly cannot tolerate more than a +3°C rise before yields decline (Easterling et al. 2007). In view of these predictions, it is sobering that model simulations project worldwide temperature increases of 4–5°C by 2080 (Easterling et al. 2007). Improvement of predictions of the effect of temperature increase on crop yields requires better models to explain plant responses to temperature change. This will also depend on our ability to improve our experimental design to simulate the natural environment. Recent work suggests that current experimental data underpredicts the effects of warming on plant phenology in terms of spring leaf emergence and flowering time (Wolkovich et al. 2012). Plants are naturally equipped with mechanisms to respond to fluctuations in ambient temperature, as detailed elsewhere in this volume (Chapters 2 and 4), but we believe that the complex relationships between temperature and other environmental factors remain ill defined.

The importance of the circadian clock for fitness requires that it be reliable and accurate. This presented a particular puzzle for plants and poikilothermic animals whose internal temperatures vary in response to the environment, because a temperature-dependent clock would inevitably be inaccurate. Pittendrigh (1954) articulated the dilemma and performed a set of classic experiments with the *Drosophila pseudoobscura* where he measured eclosion rhythms and showed that the period was maintained at 24 h over a range of temperatures, from 16°C to 26°C. A similar lack of temperature sensitivity was observed for the phototactic response of the single-celled green alga *Euglena gracilis* for temperatures ranging from 16°C to 33°C (Bruce and Pittendrigh 1956). These early 'compensation' experiments led to the hypothesis that the clock must be temperature 'independent'. However, Hastings and Sweeney (1957) noticed that although the luminescence rhythm in the dinoflagellate *Gonyaulax polyedra* (recently renamed *Lingulodinium polyedrum*) was maintained with an average of 25.5 h, the length of the period was slightly longer at higher temperatures and shorter at lower temperatures. They concluded that, rather than independent of temperature, the clock was compensated (buffered) against temperature changes. The temperature coefficient or Q_{10} is a measure of the rate of change of a system following a 10°C increase in temperature. For typical biochemical reactions, this ranges from 2–3 (Ruoff 1992). However, the Q_{10} for circadian period was much less than 2, indicating compensation. For the *Gonyaulax* luminescence rhythm, Q_{10} was less than 1.0, suggesting 'overcompensation' (Figure 6.2A)

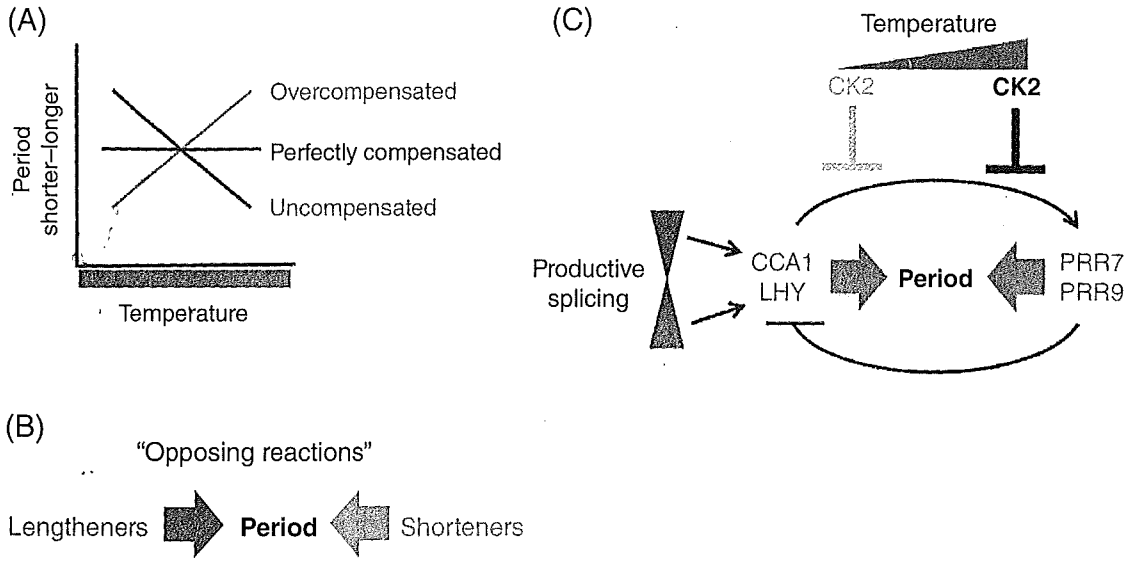

Figure 6.2 Temperature compensation relies on a balance of reactions. (A) Schema illustrating the period length associated with compensated (*black*), overcompensated (*red*), and uncompensated (*blue*) clocks over a range of temperatures. A wild-type clock that is perfectly compensated across all physiological temperatures maintains a constant period. (B) The 'Opposing Reactions' model of temperature compensation. The period is maintained relatively constant by the balance of period lengtheners and period shorteners. (C) An example of this balance is shown with CCA1/LHY and PRR7/PRR9 that are tightly regulated by several temperature-sensitive mechanisms such as CK2 phosphorylation and AS. The *cca1 lhy* double mutant has a short period suggesting CCA1 and LHY are period lengtheners, whereas the *prr7 prr9* double mutant has a long period suggesting they function as period shorteners. A balance of activities of these genes results in a compensated clock. For color detail, please see color plate section.

(Hastings and Sweeney 1957). This observation suggested that an active temperature-compensation mechanism was a natural feature of the endogenous biological clock.

One of the most confounding aspects of temperature compensation is how the temperature dependence of kinetic-rate constants, as defined by the Arrhenius equation (Bodenstein et al. 2011), can be reconciled with this clock property. A view reflecting a balancing of reactions with multiple feedback processes quickly became the model for interpreting the temperature effect on the clock (Hastings and Sweeney 1957; Karlsson and Johnsson 1972; Hoskins and Moore 2012). Perhaps one of the more detailed models described by Njus et al. (1974) postulated that ion oscillations and the regulation of transmembrane ion flux accounted for the feedback control necessary to explain the light and temperature responses. According to this model, changes in membrane lipid composition take several hours, thereby accounting for the delay in clock compensation following a temperature pulse (Njus et al. 1974). Plasma membrane viscosity alters with changes in temperature (Martinière et al.

2011), confirming a link between temperature and lipid composition. In addition, Ca^{2+} ion oscillations serve as a circadian-regulated signal that drives important processes in the plant (McAinsh et al. 1995; Trewavas 1999; Wood et al. 2001; Love et al. 2004). However, it seems that additional regulatory mechanisms have been recruited to implement temperature compensation.

Quantitative Trait Locus (QTL) studies of recombinant inbred line (RIL) populations derived from parents with similar clock characteristics under normal temperature conditions reveal transgressive segregation of circadian period and phase, meaning greater variation among the RILs than seen in the RIL parents (Swarup et al. 1999; Michael et al. 2003b). Transgressive inheritance suggests that the trait in question is controlled by additive effects of alleles at multiple loci. The architecture of the clock as multiple interlocked feedback loops is consistent with this interpretation. Loss of function of most clock genes alters period length rather than confers arrhythmia (Harmer 2009); consequently most clock genes can be thought of as period lengtheners (loss of function shortens period) and shorteners (loss of function lengthens period) (Figure 6.2B).

Similar logic can be applied to the analysis of temperature compensation. Natural variation studies in *Arabidopsis* suggest a complex and diverse genetic basis to temperature compensation. QTL analysis of several RIL populations in *Arabidopsis* suggests that multiple loci acting in *trans* are involved in regulating the levels of clock proteins to balance the response to changes in temperature (Edwards et al. 2005). Identifying the genes contributing to these *trans*-QTLs is a challenge using classical QTL mapping techniques and often requires additional analyses. One of the many *Arabidopsis* QTLs for period length that mapped only at 27°C was identified as *FLOWERING LOCUS C* (*FLC*) (Edwards et al. 2005), which encodes a MADS box transcription factor that inhibits flowering (Michaels and Amasino 1999; Sheldon et al. 1999). FLC has been shown to lengthen period in a dose-dependent manner, along with several other genes in the flowering time pathway (Salathia et al. 2006). Further transcriptomic and expression analysis implicated LUX as a candidate for mediating the effect of *FLC* on the clock at higher temperature (Edwards et al. 2005, 2006). It is likely that additional genes are contributing to this effect as predicted by the *trans*-QTLs.

A similar natural variation study was performed in *Brassica rapa* where 50 natural accessions and an RIL population of 159 individuals were screened for period length, phase, and amplitude at three temperatures. A wide variation in period length was observed with an inverse relationship between Q_{10} and period length, although Q_{10} values fell between 0.8 and 1.2, demonstrating temperature compensation in *B. rapa*. In addition,

variation in period length was more extreme in the RILs compared to the accessions, suggesting that these period extremes are selected against in natural populations (Lou et al. 2011). These results further support the connection between a synchronized clock and fitness.

To uncover the mechanistic basis for temperature compensation, a number of studies have taken a genetic approach to identify mutants that are affected in this property. The discovery of mutants affected in period length led to a deeper understanding of the link between temperature compensation and the circadian clock. Several long-period mutants in *Neurospora* and *Drosophila* show temperature-compensation defects; some maintain temperature compensation but across a more narrow range of temperatures than the wild type; other mutants were found to have fully lost temperature compensation (Gardner and Feldman 1981; Huang et al. 1995; Matsumoto et al. 1999).

In *Arabidopsis*, genetic studies have focused on the role of the clock genes in the temperature response of the circadian clock. Gould et al. (2006) showed that the transcript accumulation levels of *TOC1*, *LHY*, and *CCA1* are all temperature dependent. For example, the amplitude and peak of *TOC1* expression increased at high temperature, while *LHY* showed the reciprocal response. At lower temperatures of 12°C, *CCA1*, *LHY*, and *GI* decreased in peak expression levels (Gould et al. 2006). Salomé et al. (2010) showed that *LHY* and *CCA1* transcript levels are equally affected by temperature. At 12°C, *CCA1*, *LHY*, and *TOC1* show a phase shift, peaking later than at 22°C. At 30°C, *CCA1*, *LHY*, and *TOC1* have weak expression without a clear peak. This was surprising considering wild-type plants are fairly well compensated at this temperature (Salomé et al. 2010). Consistent with Gould et al. (2006), *CCA1* and *LHY* show low levels of expression at elevated temperature (Salomé et al. 2010). Salomé et al. (2010) observed two peaks in *TOC1* transcript accumulation at 30°C, and the level of *TOC1* expression did not exceed the peak expression level at 22°C; however, *TOC1* transcript remained relatively high at all time points and did not reach the trough levels observed at 22°C. From these studies, it is evident that there are temperature effects on the expression of these clock genes, but further studies are required to clarify these discrepancies. As discussed later in this chapter, alternative splicing (AS) also contributes to temperature-dependent transcript regulation (James et al. 2012). It may prove to be more informative to examine the temperature effects on protein levels in order to interpret the functional implications of these changes (Greenbaum et al. 2003).

In addition to *CCA1*, *LHY*, and *TOC1*, there is a clear involvement of *GI* in the temperature-compensation mechanism (Gould et al. 2006). The exact role of *GI* in this context is uncertain due to the complexity of accumulated genetic data (Rédei 1962; Huq et al. 2000; Mizoguchi et al. 2005; Gould

et al. 2006; Martin-Tryon et al. 2007; Sawa et al. 2007; Sawa and Kay 2011). Genetic screens over the years have identified a series of *gi* alleles showing a range of developmental phenotypes. As mentioned earlier, the first alleles were identified due to their severe late-flowering phenotypes (Rédei 1962), and many additional alleles have been identified on the basis of late flowering (Mizoguchi et al. 2005; Kim et al. 2007; Sawa et al. 2007; Sawa and Kay 2011). New *gi* alleles emerged with photomorphogenic phenotypes under red and blue light, implicating GI in light signaling (Huq et al. 2000; Martin-Tryon et al. 2007).

In addition to defects in flowering time and response to light, the available *gi* mutants exhibit altered temperature compensation. A line homozygous for one presumptive null allele, *gi-11*, had a period length at 17°C that matched with wild type but shortened relative to wild type when transferred to 12, 22, or 27°C (Gould et al. 2006). A line homozygous for a second presumptive null allele, *gi-201*, showed a slightly different response, with wild-type period lengths at both 22°C and 30°C and a slightly shorter period at 16°C (Salomé et al. 2010). This study also tested a line homozygous for a third mutant allele, *gi-1*, that has a 5-bp deletion leading to a premature stop codon, causing a loss of 171 amino acids from the C-terminus. This *gi-1* mutant had the same period as *gi-201* at 16°C but became increasingly shorter at higher temperatures, showing a much stronger phenotype than the presumptive null alleles (Salomé et al. 2010). The discrepancies between these two studies might reflect differences among alleles but might also be attributed to the different output rhythms monitored for assaying period length. Gould et al. (2006) measured cotyledon movement and therefore likely measured clock activity in the petiole, whereas Salomé et al. (2010) measured the luciferase activity of both *CCA1pro::Luc* and *TOC1pro::Luc* reporters in the cotyledons (e.g., Salomé et al. 2008). This may suggest tissue-specific clocks that are buffered differently to environmental signals. For example, the circadian clock in root tissue shows a different oscillation pattern than the clock in the shoot (Thain et al. 2000; James et al. 2008). In addition, certain clock output genes have different levels of temperature sensitivity leading to differential phase shifting, suggesting regulation by multiple circadian oscillators (Michael et al. 2003a).

A common trend among several mutants showing altered temperature response is conditional temperature sensitivity. Several *gi* mutants show normal period lengths at low temperature but become sensitive to high temperature. A similar trend is observed with members of the *PRR* family. The *prr7 prr9* mutant showed a wild-type period length at 12°C, but period lengthened at higher temperatures, demonstrating overcompensation (Salomé et al. 2010). In contrast, the *prr5 prr7* mutant had a slightly shorter period than wild type at 17°C and 22°C and became even shorter at 30°C.

These two observations suggest that members of the *PRR* family contribute differentially to the temperature response (Salomé et al. 2010). In addition, RVE8, involved in a negative feedback loop with PRR5, also contributes to the temperature response. Overexpression of RVE8 resulted in a short period at 17°C, 22°C, and 27°C but not at 12°C, while the *rve8-1* null mutant showed longer periods at 22°C and 27°C, but not 12°C or 17°C (Rawat et al. 2011). This suggested that PRR5 and RVE8 are important for the high-temperature-compensation response. *LHY*, *TOC1*, and *ZTL* loss-of-function mutants also showed increased sensitivity to changes in temperature; *lhy-20* and *toc1-101* are short-period mutants and ran shorter at 30°C compared to 12°C, while the long-period *ztl-4* mutant lengthened period at lower temperature (Salomé et al. 2010). These studies suggested that different compensatory mechanisms might be involved in regulating the high- *versus* low-temperature response.

The morning loop components CCA1, LHY, PRR7, and PRR9 play an important role in the temperature-compensation mechanism (Figure 6.2C). As mentioned, high temperature causes period lengthening in the *prr7 prr9* mutant. Temperature compensation is restored following an miRNA-induced reduction of *CCA1* and *LHY* expression, demonstrating that the role of PRR7 and PRR9 at high temperature is completely dependent on *CCA1* and *LHY* (Salomé et al. 2010). In addition, reduction of *CCA1* and *LHY* expression in wild type and *prr7 prr9* resulted in a short-period phenotype at all temperatures, suggesting that the role of PRR7 and PRR9 in temperature compensation is the regulation of *CCA1* and *LHY* (Salomé et al. 2010). This is consistent with PRR7- and PRR9-dependent repression of *CCA1* and *LHY* (Farré et al. 2005; Nakamichi et al. 2010). CCA1 and LHY complete the feedback loop by positively regulating *PRR7* and *PRR9* expression (Farré et al. 2005).

The binding affinity of CCA1 to the promoters of both *PRR7* and *PRR9* increased at higher temperatures, and this was inhibited by the protein kinase CK2-dependent phosphorylation of CCA1 (Sugano et al. 1998; Daniel et al. 2004; Portolés and Más 2010). CK2 activity increased at high temperature and caused accumulation of phosphorylated CCA1 and possibly a reduction in CCA1 binding to *PRR7*, *PRR9*, *TOC1*, and *LUX* promoters. The reduction in activity of CCA1 would explain the observed misexpression of *TOC1pro::Luc* transcription at high temperature (Gould et al. 2006; Portolés and Más 2010). This mechanism is reminiscent of the CK2-dependent phosphorylation of the FREQUENCY (FRQ) clock protein in *Neurospora*. Unlike CCA1, the phosphorylation of FRQ led to its degradation, but the involvement of CK2 at high temperature was consistent in both systems (Mehra et al. 2009). However, it should be noted that Portolés and Más (2010) performed promoter binding and phosphorylation assays using overexpression versions of CCA1 and CKB4,

the regulatory subunit of CK2. The potential for overexpression to introduce artifactual protein–protein interactions makes it problematic to use these results to extrapolate to the relationship between CCA1 and CKB4 circadian-regulated accumulation and temperature in wild-type plants.

CCA1 protein was shown to accumulate just before dawn and reached peak levels 1 h after dawn (Wang and Tobin 1998). Due to the circadian regulation of each of these proteins, it is difficult to predict the importance of the CKB4-CK2-dependent phosphorylation of CCA1 at high temperature without detailed information regarding CKB4-CK2 protein levels. CKB4 protein levels were found to be circadian regulated and began to accumulate near the end of the day (Perales et al. 2006). At normal temperatures, phosphorylation of CCA1 by CK2 is required for formation of CCA1–DNA complexes (Daniel et al. 2004), suggesting that the phospho-dependent regulation of CCA1 is pervasive. This was the case for FRQ in *Neurospora* where 75 temporally regulated phosphorylation sites have been uncovered where phosphorylation either stabilized or destabilized FRQ (Baker et al. 2009). Therefore, it is possible that CK2-dependent phosphorylation of CCA1 may lead to activation or repression of activity based on the temporal and spatial conditions, as well as the sites of phosphorylation. Additional studies are required to further characterize the phase-specific phosphorylation of CCA1 and the impacts of changes in temperature.

It appears that the interplay among multiple regulatory loops is important for proper buffering in response to temperature changes, and this involves a cascade of molecular players as evident from both genetic and natural variation studies. Ultimately, the controlled regulation of period lengtheners and shorteners, for example, CCA1/LHY and PRR7/PRR9, respectively, is necessary for proper temperature compensation. The type of regulation imposed on these factors appears to be dependent on the direction of the temperature change (Figure 6.2C).

6.3 Temperature entrainment

Following the discovery of an endogenous circadian clock, inquiries arose about the properties of the clock and whether inputs from the environment are in fact necessary for maintaining the correct circadian oscillations. As early as 1832, de Candolle (de Candolle 1832) established that the rhythm in leaf movement could be inverted by reversing the alternation of light and dark, establishing that the clock could be entrained to new environmental cycles. Subsequent studies emphasized the role of light as an obvious *zeitgeber*. Light pulses were capable of shifting the phase of the clock. Moreover, the magnitude and direction (advances *versus* delays) of the

resultant phase shift was dependent on the time of day at which the pulse was given, indicating that the clock modulates its own sensitivity to phase-shifting stimuli (Pittendrigh et al. 1958; Pittendrigh and Bruce 1959). In this way, the term 'temperature entrainment' addresses how temperature acts as a *zeitgeber* to set the phase of the clock. The ability to entrain the circadian clock by temperature also offers a way to delineate the contributions of clock components by examining their response to light *versus* temperature cycles.

Surprisingly, it appears that thermal entrainment is under distinct genetic regulation compared to photic entrainment. A comparison of photic and thermal entrainment on an *Arabidopsis* RIL population revealed nonoverlapping QTL for the two entrainment conditions, suggesting distinct allelic control (Boikoglou et al. 2011). In order to demonstrate temperature entrainment, organisms are grown under LL with temperature cycles. In the case of *Arabidopsis*, an example of a thermocycle commonly used is 12 h at 22°C and 12 h at 16°C (Boikoglou et al. 2011). Although a temperature difference of as little as 4°C has been shown to be sufficient for temperature entrainment (Michael et al. 2003a), the lower limit of this sensitivity has not been determined. Plants entrained in thermocycles and shifted to constant temperature continued to oscillate based on the original temperature phase as observed with light entrainment (Somers et al. 1998). The phase of entrainment in response to temperature cycles is typically the warm temperature that is interpreted as the light part of the cycle, such that light-expressed genes, including *CCA1* and *LHY*, peak early in the warm part of the cycle. Period mutants that properly entrain to temperature cycles show the expected phase relationships where short-period mutants exhibited a leading phase and long-period mutants showed a lagging phase during entrainment (Salomé and McClung 2005). Mutants that were deficient in temperature entrainment did not anticipate the warm–cold or cold–warm transition. The *toc1-2* and *lhy-20* short-period mutants showed a leading phase after 5 days at 22°C LD entrainment with release into 22°–18°C thermocycles in phase with the preceding photocycles (22°C replaces the light; 18°C replaces the dark). Similarly, the *ztl-4* long-period mutant exhibited a lagging phase upon transfer to thermocycles (Salomé and McClung 2005). These results suggested that these mutants were affected in their ability to be entrained by both light and temperature cycles.

Further examination of other clock mutants revealed a role for members of the PRR family in temperature entrainment. The *prr7-3*, *prr5-3*, and to a lesser extent *prr9-1* single mutants all showed defects in their ability to entrain to thermocycles. The *prr3-1* mutant was able to entrain to thermocycles after being initially entrained to photocycles (Salomé and McClung 2005). These entrainment assays were performed using leaf movement

data, and given the discrepancies that have been observed in the *GI* alleles (Gould et al. 2006; Salomé et al. 2010) comparing leaf movement and luciferase activity, further studies using luciferase reporters in these *PRR* mutants are needed.

Strikingly, the *prr7-3 prr9-1* double mutant was found to have an extremely long period of approximately 30–36 h (Farré et al. 2005; Salomé and McClung 2005). The double mutant following temperature entrainment of hot/cold, LL into hot/hot, and LL (HC, LL into HH, LL) exhibited arhythmicity in cotyledon movement and *CCA1* or *TOC1* promoter luciferase experiments. Occasionally the first peak of *CCA1* or *TOC1* expression occurred but was not maintained (Salomé and McClung 2005; Salomé et al. 2010). Since the *prr7-3 prr9-1* mutant can be entrained by LD cycles (Farré et al. 2005; Salomé and McClung 2005), the response to temperature could be tested by transferring photocycle-entrained plants to a HC and LL cycle that is in phase with the photocycle. Under these entrainment conditions, *CCA1* and *LHY* promoter expression peaked later than wild type but showed mild anticipation of the temperature transition. The *TOC1* promoter showed a different expression pattern with a peak 12 h earlier than in wild type, and no anticipation of the temperature transition was observed (Salomé and McClung 2005). Not surprisingly, the triple *cca1 lhy toc1* and quadruple *prr5 prr7 prr9 toc1* mutants were unable to entrain to LD or HC cycles (Ding et al. 2007; Yamashino et al. 2008). This emphasized the importance of PRR7 and PRR9 regulation of *CCA1* and *LHY* expression as well as a connection between proper phasing of *TOC1* expression and the ability to maintain temperature entrainment.

Two additional members of the PRR family that have not been thoroughly characterized, but may be important in temperature integration into the clock, are PRR3 and PRR5. Salomé and McClung (2005) included the single mutants of these genes in their temperature entrainment assays, and it was clear that *prr3-1* showed a mild lagging phase phenotype following thermocycle entrainment. However, *prr5-3* had a strong entrainment phenotype, especially when seedlings entrained by photocycles were transferred into a thermocycle (HH, LD into HC, LL), suggesting a defect in the ability to be entrained by temperature. Given the similar trend in phenotypes in these two mutants, it would be worthwhile to test temperature entrainment in the *prr3 prr5* double mutant. The *PRR* genes each showed peak expression at different times of day. *PRR9* peaked at dawn, *PRR7* in the morning, *PRR5* at midday, and *PRR3* and *TOC1* at the end of the day (Matsushika et al. 2000; Mizuno 2004). Although *PRR5*, *PRR7*, and *PRR9* are thought to have some redundant function, their single-mutant phenotypes, as well as their expression patterns, suggested that they contribute differentially to the temperature-response mechanism

(Mizuno 2004; Nakamichi et al. 2010). To define the contribution of these proteins will require further studies, in particular information on the abundance and activities of these proteins during temperature entrainment.

Further insight into temperature entrainment has arisen through the studies of ELF3, a member of the EC. Loss of ELF3 function conferred a number of phenotypes, including early flowering, photoperiod insensitivity, altered photomorphogenesis, and a long hypocotyl under white light (Zagotta et al. 1992). All of these phenotypes involve processes that are tightly linked to the circadian clock (Yakir et al. 2007). Leaf movement analysis in *elf3* did not detect rhythmic movement under LL. A luciferase reporter using the promoter of chlorophyll *a/b* binding protein gene (*CAB2*) confirmed the lack of a rhythm in *elf3* under red and blue light (Hicks et al. 1996b). The *elf3* mutant was disrupted in temperature entrainment and was unable to maintain rhythmicity following 4°C or 10°C thermocycle differences (McWatters et al. 2000; Thines and Harmon 2010).

Wild-type plants that are entrained to 24 h thermocycles and transferred to shorter cycles, for example, 6 h warm/6 h cold, show a 24 h periodicity where every other peak is greater than the 6 h peak. This type of experiment is known as frequency demultiplication and tests whether the plant is simply responding to the alternation between warm and cold (LD) or instead is still manifesting a functioning endogenous circadian oscillator. Mutants with nonfunctional oscillators do not show any signs of their previous 24 h entrainment and instead peak in phase with alternating external cues (McWatters et al. 2000; McWatters et al. 2007). This was observed for *elf3* under 12 h thermocycles, demonstrating the importance of ELF3 for proper temperature entrainment (Thines and Harmon 2010). Interestingly, overexpression of ELF3 did not affect the phase response following resetting by temperature cues, implying that ELF3 was not important for the ability of the clock to perceive temperature (Thines and Harmon 2010). This observation not only established ELF3 as a target of temperature input to the clock but also distinguishes mechanisms of perception and response to temperature.

The temperature induction of *PRR7*, *PRR9*, and *GI* mRNA abundance was eliminated in the *elf3* mutant, and the basal levels of expression of these genes were elevated, especially at night (Thines and Harmon 2010). The elevated expression at night was consistent with the normal role of the EC in gating the induction of these genes by repressing expression at night (Nusinow et al. 2011). It appears that the role of ELF3 is not specific to temperature entrainment, but, rather, ELF3 is necessary to maintain proper circadian clock function (Herrero et al. 2012) and regulation of temperature-responsive clock genes (Thines and Harmon 2010).

6.4 Cold tolerance

Temperate plants rely on seasonal cues from the environment, such as day length and temperature, to signal impending changes in growth conditions (Rohde and Bhalerao 2007; de Montaigu et al. 2010). Cold plays a major role throughout the plant life cycle from stratification, the cold-induced breaking of seed dormancy, to vernalization, the cold-induced process that conditions the plant to respond to flower-inducing stimuli. In order to survive the winter season, plants must undergo a process called cold acclimation. The gradual decline in temperature exposes the plant to nonfreezing temperatures. This triggers an increase in cold tolerance, allowing the plant to survive temperatures that would be lethal without this preconditioning. Due to the pressures to maximize the growing season for crops despite the threat of freezing temperatures, considerable attention is focused on understanding how plants respond to the cold (Thomashow 1999).

As highlighted in Chapters 2 and 5, the cold-response pathway involves three members of the AP2/ERF family of transcription factors referred to as CBF1–CBF3/DREB1B, DREB1C, and DREB1A. These genes are tandemly linked in *Arabidopsis* and induced within 15 min of exposure to low temperature (Stockinger et al. 1997; Gilmour et al. 1998; Liu et al. 1998). CBFs bind to C-repeat/dehydration-responsive elements (CRT/DRE) in the promoters of more than 100 cold-responsive genes, commonly referred to as the CBF regulon (Shinozaki and Yamaguchi-Shinozaki 2000; Vogel et al. 2005). The induction of these CBF-targeted genes is sufficient to induce freezing tolerance, because constitutive expression of the CBF genes induced freezing tolerance in the absence of a low-temperature treatment (Jaglo-Ottosen et al. 1998; Liu et al. 1998). Among the cold-induced genes are the *COR* genes that encode hydrophilic polypeptides, in particular *COR15a*, that are important for the cryoprotection of the plasma membrane during freeze tolerance (Steponkus et al. 1998). The CBF pathway is also involved in cold-induced changes in the metabolome that lead to the synthesis of cryoprotectants (Cook et al. 2004; Kaplan et al. 2004).

Microarray studies of the circadian-regulated transcriptome in *Arabidopsis* revealed a circadian oscillation in the pattern of expression of *CBF3* with a peak at midday (Harmer et al. 2000). Later studies showed that *CBF1* and *CBF2* are also circadian regulated, along with two other cold-responsive genes, *RAV1* and *ZAT12*, suggesting a role for the clock in modulating the response to low temperature (Fowler et al. 2005). The consequence of the circadian regulation of the *CBF* genes is a time-of-day-dependent effect on low-temperature-induced transcript accumulation, shown to result in an increase in cold induction during the day compared to the night (Fowler et al. 2005; Dong et al. 2011). The rhythmicity of *CBF1* and *CBF3*, as well as several *COR* genes, was lost in the *cca1 lhy* double mutant, while the

CBF2 amplitude was dramatically reduced. The effect of these disruptions in cold-responsive gene expression was a 50% reduction in freezing tolerance in *cca1 lhy* based on electrolyte leakage and seedling survival assays (Espinoza et al. 2010; Dong et al. 2011).

Consistent with the role of the PRRs in the temperature-compensation mechanism, the *prr5 prr7 prr9* mutant was shown to be significantly more resistant to freezing tolerance than wild type such that 62% of the mutants survived a 1-day −5°C cold treatment that was completely lethal to wild-type plants (Nakamichi et al. 2009). Not surprisingly, this arrhythmic mutant was affected in many processes regulated by the clock such as resistance to salt and drought stress as well as misexpression of a large suite of genes involved in a range of processes from abiotic and biotic stress to metabolism and development (Nakamichi et al. 2009).

Evidently, the circadian clock tightly controls the cold-response pathway as seen by the phenotypes of these clock mutants. However, another important question to address is how the clock itself responds to low temperature. Bieniawska et al. (2008) showed that in *Arabidopsis*, oscillations in steady-state mRNA abundance for many clock and clock-regulated genes were dramatically compromised at 4°C. For most transcripts, the rhythmic amplitude was greatly attenuated in LD and virtually abolished in LL. Abundance of most, but not all, transcripts dampened to high levels. The transcript of the clock gene *LUX* retained a robust oscillation at 4°C in LD but showed only low amplitude oscillations in LL, and because the experiment only examined the first two cycles in LL, it was unclear if that apparent rhythmicity persisted. Not surprisingly, the cold-responsive genes that have been shown to be clock regulated, such as *CBF1* and *CBF2*, are induced following cold treatment and remain elevated, becoming arrhythmic in both LD and LL (Bieniawska et al. 2008). Thus, although the clock plays a crucial role gating the transcriptional response to cold, clock function is greatly compromised if not completely abolished at 4°C, with the acknowledged caveat that

Bieniawska et al. (2008) only examined the first two cycles following onset of cold. The ability of *Arabidopsis* to persist for extended periods at cold temperatures raises the question of whether clock function might recover to some extent during extended cold treatment.

In addition to being involved in the response to a rapid drop in temperature, the circadian clock is also important under extended cold conditions to enable proper endodormancy in trees such as chestnut, *Castanea sativa* Mill. (Ramos et al. 2005). Endodormancy is the deep dormancy during winter that inhibits growth until a chilling requirement is reached. Short-day conditions and low temperatures lead to growth cessation, bud set, and the start of cold acclimation. Stem tissue collected from chestnut at midday in June and December showed *TOC1* transcript in the winter sample at a

time when *TOC1* expression is normally inhibited. Further analysis revealed oscillations of *CCA1* and *LHY* mRNA under constant conditions, confirming that a circadian rhythm existed at 20°C; however, similar to *Arabidopsis*, the oscillations were abolished in the winter. This effect was not due to the endodormancy state since cold pulses on nondormant chestnut seedlings produced the same effect (Ramos et al. 2005). *PRR* homologues were identified in chestnut based on sequence similarity and expression pattern and designated *CsPRR5*, *CsPRR7*, and *CsPRR9*. Consistent with the other clock genes, the *CsPRRs* were highly expressed in winter samples with no oscillation pattern evident. Rhythmicity was regained following 1 week of warm temperatures, demonstrating a clear connection between temperature sensing, circadian clock response, and regulated plant growth (Ibanez et al. 2008).

Analysis of the metabolome suggested that a subset of metabolites showed circadian regulation based on their accumulation in LL; these included maltose, O-acetyl serine, valine, and alanine. Of these, only maltose maintained oscillation patterns at 4°C (Espinoza et al. 2010). Network modeling based on correlations between changes in metabolites under diurnal (LD) and circadian (LL) conditions at 4°C and 20°C revealed more connections at 4°C in LL and LD, implying a large coordinated shift in the metabolic profile at 4°C (Espinoza et al. 2010). In addition to changes in metabolite levels, cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) levels also responded to changes in temperature, and this was also time-of-day dependent. A low-temperature treatment of 0°C resulted in an increase in guard cell $[Ca^{2+}]_{cyt}$ that was greatest at midday (ZT6.5 h) (Dodd et al. 2006). This increase may be important for the regulation of the CBF response through a calcium-dependent calmodulin activation of CAMTA3, a regulator of *CBF1*, *CBF3*, and *ZAT12*. CAMTA3, a member of the calmodulin-binding transcription activator family, has been shown to bind to the promoters of *CBF1*, *CBF3*, and *ZAT12* and positively regulate their expression in response to cold treatment. The *camta1 camta3* double mutant showed a reduction in freezing tolerance following cold acclimation, and *CBF2* transcript levels were reduced by 50% in the *camta3* mutants following cold treatment, suggesting that this is one of several regulators of *CBF2* (Doherty et al. 2009). This provides a possible mechanism for the integration of calcium signaling through CAMTA3 and an important component to the process of freezing tolerance.

Freezing tolerance is particularly relevant to the growth and optimization of tomato, an extremely chilling-sensitive crop. Upon transfer to low temperatures, tomato plants showed a sharp reduction in chloroplast function, leading to a decrease in photosynthesis due to limits on carbon availability. Cold treatment also resulted in a suspension in circadian regulation of transcription (Martino-Catt and Ort 1992). Interestingly, the

CBF pathway is slightly modified in *Solanum* species. Although the *CBF* genes show a similar structural organization, the locus is fairly divergent across species. Tomato (*Solanum lycopersicum*) has *CBF1–3* tandemly arranged similarly to *Arabidopsis*; however, only *Sl-CBF1* was cold responsive and this is consistent with the high conservation of the upstream region of *CBF1* in the *Solanum* family. *Solanum CBF1* also displays circadian regulation (Pennycooke et al. 2008). Other *Solanum* species such as *Solanum tuberosum* (potato) and *Solanum commersonii* (diploid tuber-bearing potato) have copies of *CBF4* that are cold responsive. The more freezing-tolerant *S. commersonii* showed higher expression levels of *CBF1* and *CBF4* following cold temperatures compared to the more sensitive *S. tuberosum*, demonstrating a clear connection between the regulation of the *CBFs* and the plant's ability to tolerate low temperatures (Pennycooke et al. 2008).

Aside from freezing tolerance, many plants rely on the cold during the winter season to know when to flower and require vernalization to stimulate flowering in the spring (discussed in detail in Chapter 4). To prevent unwanted flowering following a brief temperature drop in the fall, vernalization requires an extended cold treatment before initializing flowering. In *Arabidopsis*, two important genes required for proper flowering time control are *FLC* and *FRIGIDA (FRI)*. *FLC* is an inhibitor of flowering and its expression is promoted by *FRI* in the fall and inhibited by vernalization (Amasino 2010). Due to the effect of altered *FLC* expression and period length, Salathia et al. (2006) examined the effect of vernalization on the circadian clock. Leaf movement analysis in various mutant backgrounds demonstrated that vernalization shortened circadian period independent of *FLC* and *FRI*, suggesting a connection between vernalization and the circadian clock (Salathia et al. 2006). One well-established result of vernalization is epigenetic changes in chromatin structure at the *FLC* locus (Kim et al. 2009; Amasino 2010; Crevillén and Dean 2011). Accumulating data indicate that rhythmic chromatin changes are associated with cycling clock gene expression (Perales and Más 2007; Jones et al. 2010; Farinas and Mas 2011; Lu et al. 2011). One possibility is that vernalization induces analogous epigenetic changes at one or more clock gene loci, with consequent changes in clock period (Sheldon et al. 2009; Deng et al. 2011). These changes in clock period under constant conditions translate to a phase shift under diurnal conditions. The clock regulates the phase of the response relative to the LD cycle, resulting in gated control of output pathways as demonstrated by the distinct phases of peak hypocotyl growth in LL and LD conditions (Dowson-Day and Millar 1999; Nozue et al. 2007). Although period estimates are important for drawing conclusions about the involvement of the circadian clock, the associated phase is directly applicable to the diurnal growth of the plant seen in nature.

6.5 Splicing

The role of AS in the circadian clock is becoming increasingly recognized and evidence is accumulating for thermosensitivity of splicing events. In *Neurospora*, two forms of the clock protein FRQ generated via AS are present at ratios that are temperature dependent. These AS events are thought to be important for controlling the response to ambient temperature (Liu et al. 1997; Colot et al. 2005; Diernfellner et al. 2007). Similarly, the *PER* gene in *Drosophila* is alternatively spliced at low temperature, leading to an increase in PER protein earlier in the day than at normal temperatures (Majercak et al. 1999).

Recently, thermosensitive AS has been demonstrated in *Arabidopsis* for a number of clock genes. Two alternatively spliced isoforms with premature termination codons for *CCA1* accumulated differentially at low temperature (Filichkin et al. 2010; Filichkin and Mockler 2012). James et al. (2012) extended this analysis using a high-resolution RT-PCR system to detect AS events for *CCA1*, *LHY*, *TOC1*, *PRR3*, *PRR5*, *PRR7*, *PRR9*, *GI*, *ZTL*, and *CHE*. They identified 63 new splicing events for these clock genes, several of which were shown to be temperature dependent. In the case of *CCA1* and *LHY*, a shift from 20°C to 4°C at dusk resulted in an increase of unproductive AS products for *LHY* and a decrease for *CCA1*. Consistent with these results, CCA1 protein levels were much higher than those of LHY following a shift to cold temperatures relative to 20°C. Similar opposing patterns of AS product accumulation were observed for *PRR7* and *PRR9* (James et al. 2012). Although one splicing factor, PRMT5, has been shown to be important for the AS of *PRR7* and *PRR9* (Deng et al. 2010; Hong et al. 2010; Sanchez et al. 2010), it is not known whether this process is temperature sensitive. The exact consequence of these AS events on protein abundance has not been thoroughly explored; future studies examining protein accumulation at various temperatures are needed.

Seo et al. (2012) proposed a mechanism for CCA1 protein regulation through an alternatively spliced *CCA1* transcript containing an early stop codon in the retained fourth intron. They suggested that an ATG following the premature stop codon leads to translation of a truncated protein, CCA1ß, that lacks the MYB DNA-binding domain but retains the dimerization domain necessary for interaction with CCA1 and LHY. This truncated CCA1ß protein interacted with full-length CCA1 and LHY in yeast and inhibited target promoter binding. Overexpression of CCA1ß *in planta* shortened circadian period, consistent with the truncated CCA1ß acting as a negative regulator of CCA1 and LHY activity; however, it has yet to be demonstrated directly that this truncated CCA1ß protein is present in plants (Seo et al. 2012). Consistent with previous studies (Gould et al. 2006), Seo et al. (2012) confirmed the temperature-dependent ratio of

CCA1 splice transcripts with a decrease in the inhibitory splice variant at low temperature, suggesting greater activity of CCA1 protein.

The presence of nonfunctional transcripts is important to consider when interpreting measurements of total transcript levels. In the case of James et al. (James et al. 2012), circadian oscillations were maintained at 4°C and transcript levels for *CCA1* and *LHY* were reduced, whereas Bieniawska et al. (2008) showed that the oscillations were severely reduced but transcript levels remain high. Other discrepancies in the pattern of expression of clock genes in response to temperature changes have been mentioned previously, highlighting the complexity of the transcriptional regulation of these genes. Future studies on the functional implications of temperature on circadian clock genes need to address both transcript and protein level.

6.6 Concluding remarks

The impending threat of climate change has become a driving force for research into how plants sense and respond to temperature. The circadian clock is an important regulator of plant growth and likely an essential driver of the temperature response due to the role of the clock in the coordination of important metabolic, physiological, and behavioral processes with the external environment. It has been recently shown that metabolism and growth are temperature compensated, although the mechanistic details remain incompletely defined. Biomass accumulation, starch turnover, and protein content were all unaltered with decreases in the night temperature. Variation in the overall growth rate was dependent on changes in the daytime temperature (Pyl et al. 2012). The inherent property of temperature compensation and the ability of the clock to be entrained to temperature cycles highlight the close relationship that exists between changes in ambient temperature and clock adaptability.

An important question remains: how does the clock sense a change in temperature? There are several mechanisms that contribute to the thermal response, some of which are depicted in Figure 6.3. For example, increases in temperature lead to a reduction in H2A.Z nucleosome occupancy causing a relaxation in chromatin, allowing for the expression of warm temperature-responsive genes, such as *HSP70* (Kumar and Wigge 2010). This heat-sensitive response may trigger the activation of circadian genes; the mechanism that exists for *TOC1* activation by H3 acetylation might be temperature sensitive. It seems likely that the activation (or inactivation) of temperature-sensitive enzymes like CK2 will be important for triggering a fast response through posttranslational activation (or inactivation) of clock proteins as seen with CCA1. Finally, the role of AS is an important avenue for further investigation. Low-temperature effects on *LHY* are apparent,

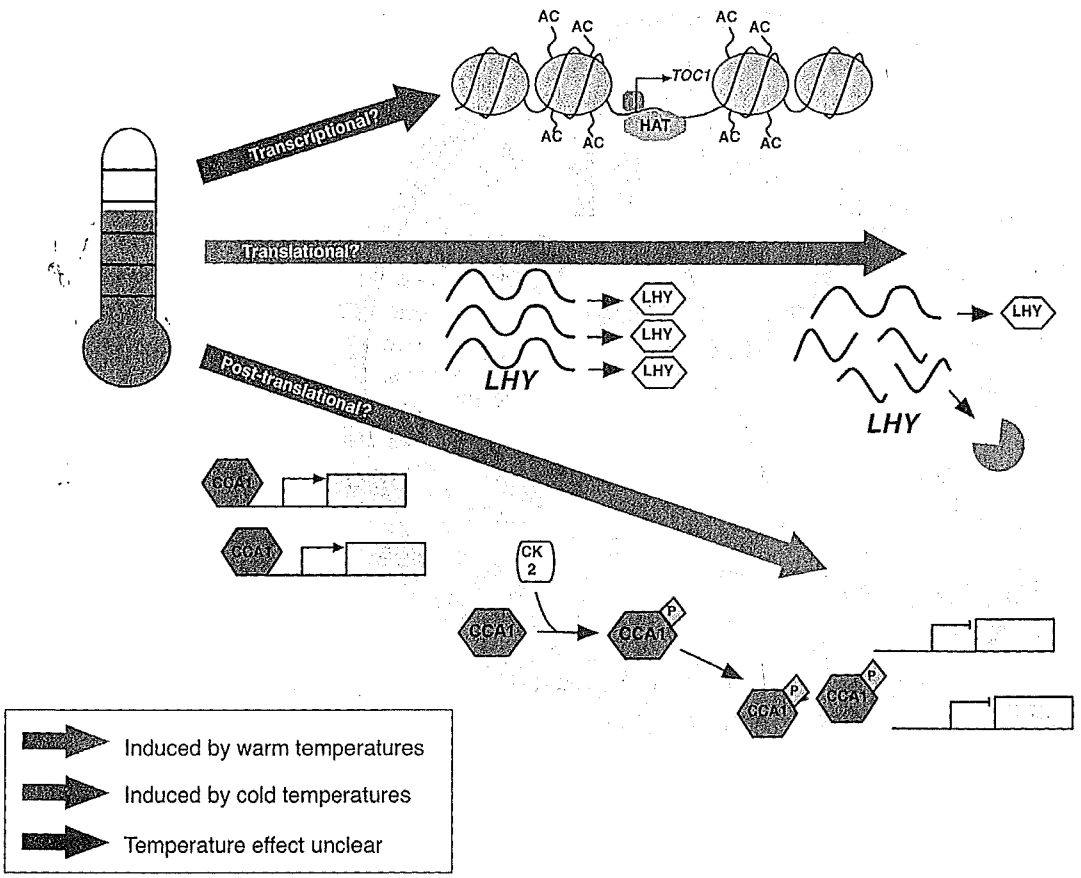

Figure 6.3 Possible points of temperature integration into the circadian clock. It is still not clear how the clock senses changes in ambient temperature. The three processes depicted in this figure integrate temperature at the level of transcriptional, translational, and posttranslational regulation. The *black arrow* represents the transcriptional regulation of chromatin remodeling that has been shown to be important for *TOC1* regulation but not yet linked to temperature. However, high temperature has been shown to deplete H2A.Z nucleosome occupancy leading to activation of the warm temperature transcriptome (Kumar and Wigge 2010). The translational arrow describes the temperature-dependent AS events for *LHY*. As the temperature cools (indicated by a transition from *red* to *blue* in the *arrow*), there is an increase in unproductive splice forms for *LHY* that are likely responsible for the decrease in protein abundance. Finally, the posttranslational arrow depicts the high-temperature-dependent (illustrated by a *blue* to *red* color transition) phosphorylation of CCA1 by CK2, leading to a decrease in CCA1 activity. The exact timing or sensitivity of this response is unclear but may be an important mechanism for fine-tuning the temperature response. For color detail, please see color plate section.

and it will be important to examine other clock genes at various temperatures to assess the implications of this regulation on protein abundance. Figure 6.3 does not address $[Ca^{2+}]_{cyt}$ although it has been known for many years that changes in $[Ca^{2+}]_{cyt}$ are among the most rapid events detected following exposure to either low or high temperatures and are likely to be close to at least one primary temperature-sensing mechanism (Knight et al. 1996; Gong et al. 1998; McClung and Davis 2010). Nonetheless, within the context of the circadian clock, it seems that $[Ca^{2+}]_{cyt}$ is used as a clock output signaling intermediate rather than as a component of an input

pathway signaling from a primary temperature sensor (Dodd et al. 2005a; Xu et al. 2007).

Significant gaps remain in our understanding of plant temperature responses and, in particular, how a clock can be temperature responsive and entrained by temperature cycles yet also buffered (compensated) against changes in temperature. Neither the primary temperature sensors nor the signaling pathways to the clock have been fully elucidated. Future studies must face the challenges of both identifying temperature signaling components and deciphering the order of events following a change in temperature in terms of the spatial and temporal regulation of the clock network.

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